ROLE OF GOLD NANOPARTICLES TOWARDS ENHANCEMENT OF ANTIMELANOMA BIOEFFICACY OF THE PLANT *MADHUCA LONGIFOLIA*: A GREEN NANOTECHNOLOGICAL PERSPECTIVE

A

SYNOPSIS

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Submitted by

SAURABH YADAV
M.Sc., M.Phil.

Dr. M. M. Srivastava
Supervisor
Professor
Department of Chemistry

Dr. N. Ganesh
Co-Supervisor
Head & Sr. Scientist,
J. N. Cancer Hospital & Research Center, Bhopal

Dr. Sahab Dass
Professor & Head
Department of Chemistry

Prof. Ravinder Kumar
Dean
Faculty of Science

DAYALBAGH EDUCATIONAL INSTITUTE
(Deemed University)
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INTRODUCTION:

In spite of excellent advancements for diagnosis and treatment, cancer is a big, silent intimidation to our society. It is the second most common disease after cardiovascular disorders for maximum deaths in the world. Skin cancers are comparatively unusual malignancies worldwide not ranking among the first ten common cancers. Recently, there has been a progressive increase in the occurrence of skin cancers. Three most common primary skin cancers are *basal cell carcinoma* (BCC), *squamous cell carcinoma* (SCC) and *malignant melanoma (American Cancer Society, Report, 2009)*. Skin cancers, particularly cutaneous melanomas represent a small but significant fraction of patients with cancer *(Wagner et al. 2001, Cummins et al. 2006).* In India, skin cancers make up about 1–2 % of all diagnosed cancers *(ICMR, 2009).* Although, the rate of skin cancers in India is lower as compared to the Western world *(Deo et al. 2005).*

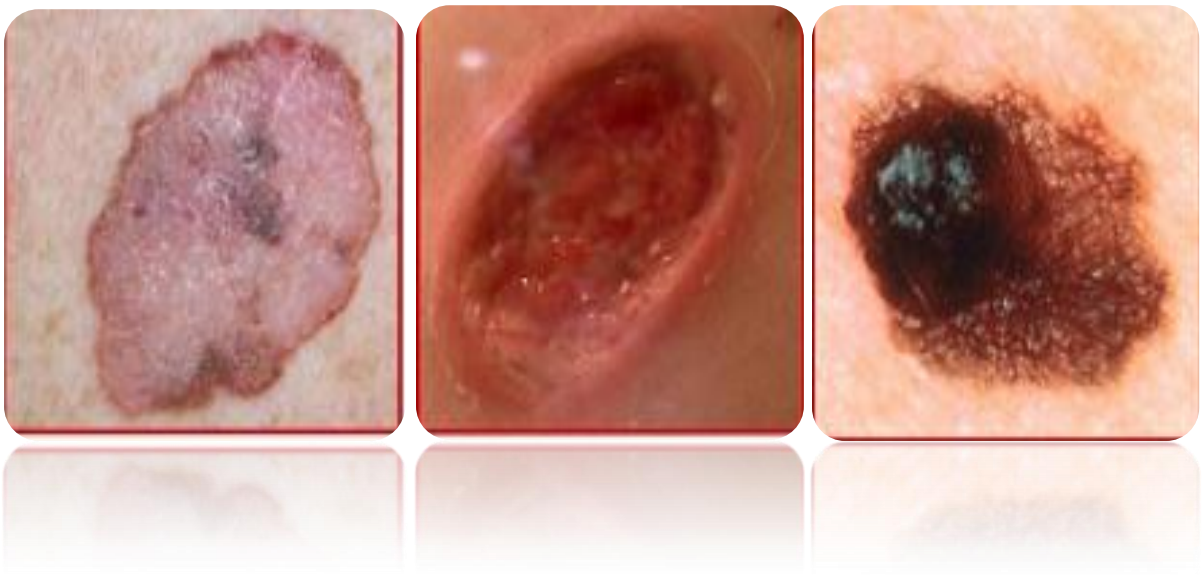


FIG. 1 PICTORIAL REPRESENTATION OF MELANOMA

Melanoma is a spiteful neoplasm of melanocytes, most often arising from the skin. Melanoma has amplified to the level of a serious public health problem during the past 20 years *(Kalkman and Baxter et al. 2004)*. Melanoma tumor is accounted for 2·6 % of the global cancer incidence and 1·1 % of cancer-related deaths *(Tramer et al. 2001, Imran et al. 2011, Ferlay et al. 2013, Siegal et al. 2013***).** Even if these data status melanoma eighth or ninth in incidence, it's doubling rate every 10–20 years is more worrying *(Hoey et al. 2007, Imran et al. 2011, Siegel et al. 2013)***.** The chemical, biological and other environmental identities are in total responsible for Indians for their high risk. The major risk factors for skin cancers is due to the exposure to ultraviolet light, which is a non- ionizing radiation *(American Cancer Society, Report 2009).* A range of risk factors for melanoma are ultraviolet (UV) light, light-colored skin, freckles, and light hair, family history of melanoma, weakened immune system *(American Cancer Society, Report, 2013*)**.** It is well thought-out as an adversary of modernization and advanced pattern of socio-cultural life dominated by Western life style.

Stages of Melanoma:

Melanoma, the most vicious form of skin cancer, is always staged. The stage of melanoma is a description of how widespread it is. Staging of cancer is important because the stage of diagnosis is the most powerful predictor of survival and make helps to melanoma treatment team to develop an appropriate treatment plan and diagnosis. The treatments are frequently changed based on the stage *(Smith et al. 2008, Liu et al. 2010)*. The different stages of skin cancer (melanoma) can be classified as follows:

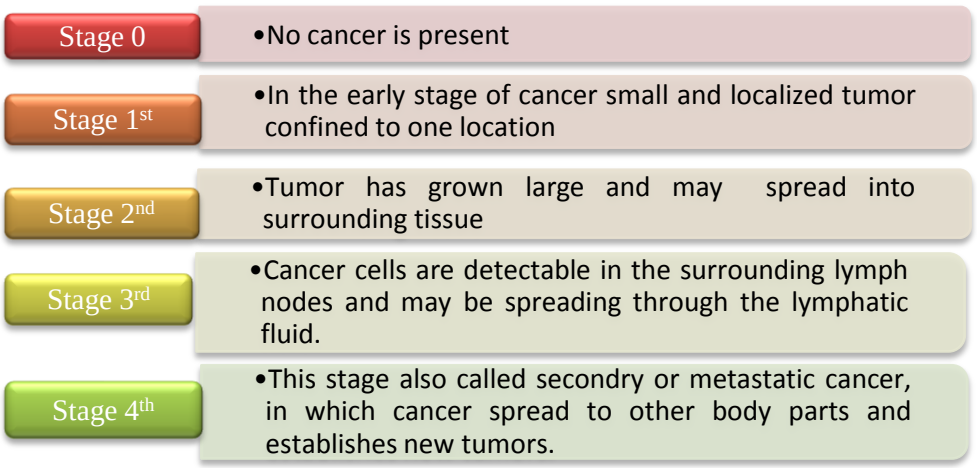

Stage 0
•No cancer is present
Stage 1st
•In the early stage of cancer small and localized tumor confined to one location
Stage 2nd
•Tumor has grown large and may spread into surrounding tissue
Stage 3rd
•Cancer cells are detectable in the surrounding lymph nodes and may be spreading through the lymphatic fluid.
Stage 4th
•This stage also called secondry or metastatic cancer, in which cancer spread to other body parts and establishes new tumors.

FIG. 2 DIFFERENT STAGES OF SKIN CANCER

Therapies used for melanoma treatment:

The various treatment modalities of melanoma are as follows:

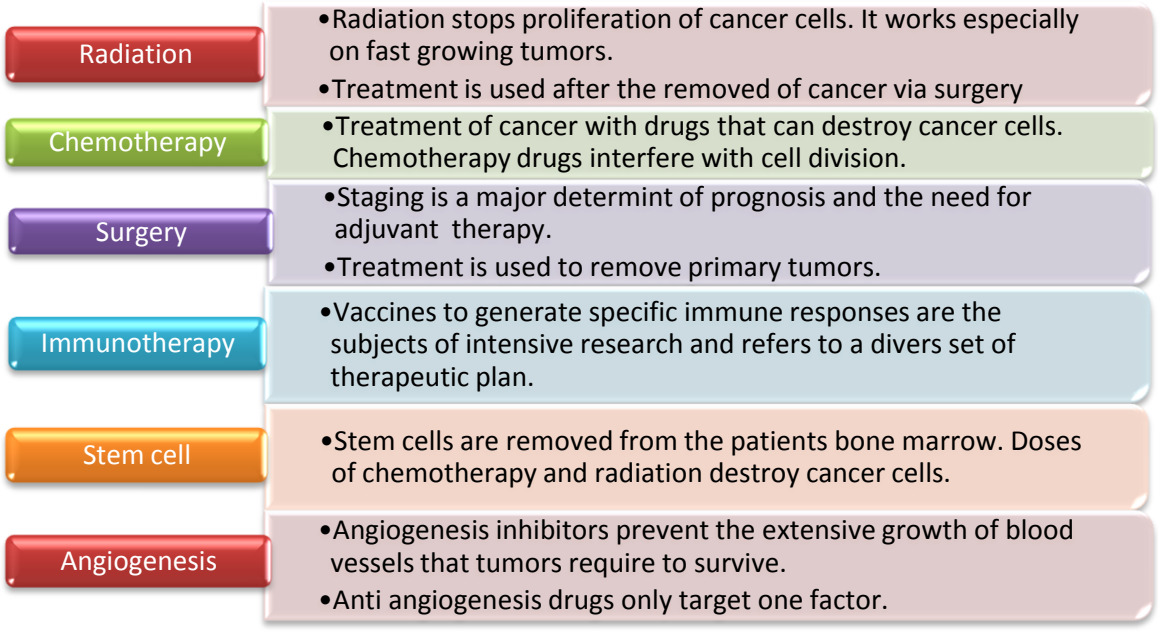
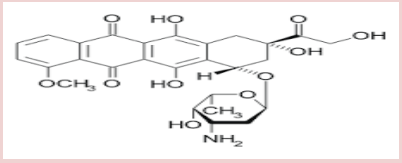
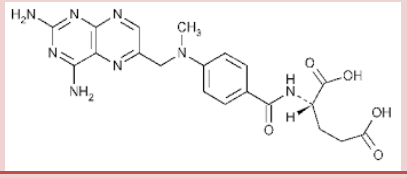
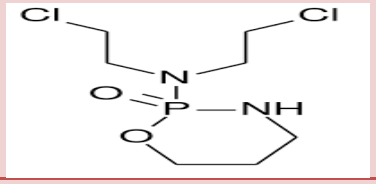
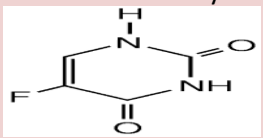
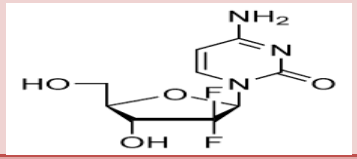
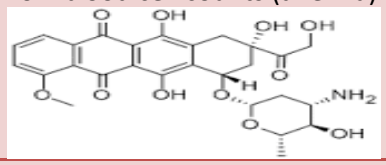
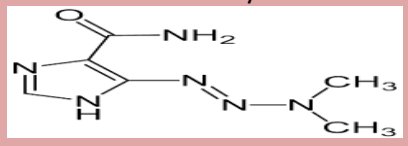
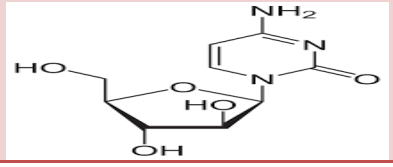

Radiation
•Radiation stops proliferation of cancer cells. It works especially on fast growing tumors.
•Treatment is used after the removed of cancer via surgery
Chemotherapy
•Treatment of cancer with drugs that can destroy cancer cells. Chemotherapy drugs interfere with cell division.
Surgery
•Staging is a major determint of prognosis and the need for adjuvant therapy.
•Treatment is used to remove primary tumors.
Immunotherapy
•Vaccines to generate specific immune responses are the subjects of intensive research and refers to a divers set of therapeutic plan.
Stem cell
•Stem cells are removed from the patients bone marrow. Doses of chemotherapy and radiation destroy cancer cells.
Angiogenesis
•Angiogenesis inhibitors prevent the extensive growth of blood vessels that tumors require to survive.
•Anti angiogenesis drugs only target one factor.

FIG. 3 TREATMENT MODALITIES OF MELANOMA

Common drugs used for melanoma with their side effects:

Chemoprevention is defined as using chemical with goal of preventing, interrupting or reversing the carcinogenic process. **"A dose of anticancer drug is sufficient to destroy tumor cells is often toxic to normal tissues and shown to many side effects, which in turn limits their treatment efficacy"** (*Kaur et al. 2007*). To tackle skin cancer a large number of chemotherapeutic drugs are known in the market. Current chemotherapy consists of cytotoxic agent which reduces the proliferative drive. There are several major issues for various chemotherapeutic drugs. **Table 1** presents the list of some common drugs used for melanoma treatment along with their side effects (*Ruther et al. 2000, Sober et al. 2001, Hijiya et al. 2007, Kaur et al. 2007*).

TABLE 1. ANTIMELANOMA DRUGS AND THEIR ADVERSE EFFECTS

Doxorubicin ❖ Allergic reactions ❖ Possible future infertility ❖ Risk of heart damage 	**Methotrexate** ❖ Major toxicity on bone marrow ❖ Causes megaloblastic anemia ❖ Bleeding in intestine 
Cyclophosphamide ❖ Irritation in the lining of urinary bladder ❖ Fever with chill 	**5-Fluorouracil** ❖ Myelosuppression ❖ Mucositis and Dermatitis ❖ Cardiac toxicity 
Gemcitabine ❖ Decrease in white blood cells ❖ Nausea and vomiting ❖ Liver infection 	**Epirubicin** ❖ Reddish urine ❖ Congestive heart failure ❖ Low blood cell counts (anemia) 
Dacarbazine ❖ Taste changes, including metallic taste ❖ Birth defects if taken during pregnancy ❖ Infertility 	**Cytrabine** ❖ Bone marrow dysfunction ❖ Lethargy, sleepiness, confusion ❖ Soreness and redness of the palms 

Source: *Carmaeia 1993, Kaur et al. 2007.*

Herbal Approach:

In the last twenty years increasing public dissatisfaction with the cost, efficacy and potential side effects of prescription and medications has developed interest in returning to natural or organic remedies *(Ruther et al. 2000 and Hijiya et al. 2007)*. Increased emphasis on the use of medicinal plants in search for new drugs is undoubtedly a productive strategy. Re-establishment of the link between plant and health is responsible for launching a new generation of plant-derived pharmaceutical *(Raj et al. 2015, Verma et al. 2014).* Over the past decade, herbal medicines have been accepted universally. Development of compounds with pharmacological efficacies from Natural Products has currently become a vital area of research. Plants are the sources of half of the pharmaceuticals in our present medicinal cabinet. Quite a lot of biologically active compounds in a plant, work mutually to produce greater effect then a single chemical *(Mendonca et al. 2006)*. The synergistic, additive or antagonistic outcome of various components of plant material may develop the therapeutic effect simultaneously dropping the side effects. Working with plant extracts, disease may not gain resistance because they are really a cocktail of active biochemical's rather than single compound. Thus, unlike compounds synthesized in the laboratory, secondary compounds from plants are almost guaranteed to have biological activity.

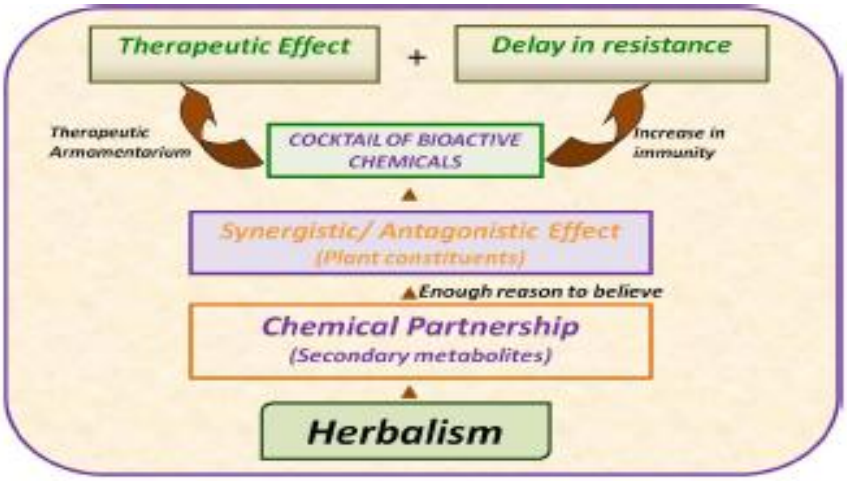


FIG. 4 PROVEN EFFICACY OF HERBALISM

Green nanotechnology:

Recent developments in nanotechnology have witnessed the fast developing power of this interdisciplinary area with countless applications in medical sciences *(Fayaz et al. 2011, Kim et al. 2012).* Green nanotechnology is an area of attention getting significant focus in current years**.** It has key intention to create nanotechnology-based products which are eco-friendly and safer for all beings. The bioactive phytochemicals implanted with nanoparticles (mostly gold nanoparticles) have a budding interdisciplinary area with potential applications of nano composites in curative applications. The phytochemicals coating on nanoparticles render non toxic quality to green gold nanoparticles *(Ahmed et al. 2015).* Thus the overall production and architecture of gold nanoparticles embedded photoproduct is highly attractive as it brings an

importance symbiosis between plant sciences and nanotechnology. Occlusion of cancer fighting phytochemicals in various plant species and their future efficacy in the development of tumor specific gold nanoparticles can be safely produced stored and shipped worldwide. Employing the concept of green nanotechnology it not only smooths the progress of the manufacturing of ecofriendly and safe products but also allow the field to grown-up with sustainable profitable feasibility *(Balachandran et al. 2005, Geethalakshmi et al. 2013).*

Green nanotechnology: The rationale

The reduction capabilities of cocktail of phytochemicals present in plants to chemically reduce gold (III) salts to gold (0) nanoparticles.

The reduction of Au (III) to Au (0) is expected to occur through the oxidation of phytochemicals having hydroxyl to carbonyl groups.

Some recent work carried out on the synthesis, characterization and evaluation of gold nanoparticles embedded with phyto extract has been summarized in

TABLE. 2 GOLD NANOPARTICLES EMBEDDED WITH PHYTO EXTRACT FOR DIFFERENT BIOEFFICACIES

S.No.	Nanoparticle	Phytochemicals study	References
1	Biosynthesis of silver and gold nanoparticle	Characterization of nanoparticles	*Huang et al. 2007*
2	Gold nanoparticles embedded with Gum	Antifungal activity	*Kattumuri et al. 2007*
3	Gold nanoparticles embedded flavonoids	Detection of biological activity	*Wang et al. 2008*
4	Nanoparticle with Flavonoid solution	Antioxidant activity	*Conec et al. 2008*
5	Gold nanoparticle embedded Soyabeans	Characterization of nanoparticles	*Shukla et al. 2008*
6	Nanoparticle with Quercetin solution	Antioxidant enzymes in cells	*Das et al. 2009*
7	Gold nanoparticle embedded flavonoid	Anticancer activity	*Katti et al. 2009*
8	Gold nanoparticle embedded tea leaf	Cytotoxic for prostate cells	*Nune et al. 2009*
9	Gold nanoparticle embedded tea leaf	Characterization of nanoparticles	*Cristina et al. 2010*
10	Gold nanoparticles with *Madhuca longifoli*	Characterization of nanoparticles	*Fayaz et al. 2011*

S.NO.	Nanoparticle	Phytochemicals study	References
11	Gold nanoparticle with *Zingiber officinal*	Characterization of nanoparticles	*Kumar et al. 2011*
12	Targeted specific Gold nanoparticles	Characterization and Applications	*Kannan et al. 2006*
13	Synthesis of Silver and gold nanoparticles	Characterization of nanoparticles	*Varahalarao et al. 2014*
14	Gold nanoparticles embedded extract	Breast cancer activity	*Smith et al. 2008*
15	Gold nanoparticles embedded Gallic acid	Cervical cancer activity	*Daduang et al. 2015*

OBJECTIVES:

The latest understanding, ***"any plant already established for some pharmacological properties should be explored for the newer bioefficacy"*** has motivated us to trap unknown anti cancer (anti melanoma) bioefficacy of the plant ***Madhuca longifolia*** with special reference to the synthesis and characterization of the gold nanoparticles embedded with the plant extracts for enhancement in the target bioefficacy.

Hypothesis:

A fact ***"Reactive Oxygen species (ROS) generated by physiological process or exogenous factors are well-known to induce oxidative break to biological macromolecule such as membrane lipids, protein and DNA which may ultimately lead to bring on cancer and other degenerative disease. Antioxidant are there, for found to be highly successful to inhibit apoptosis mediates by oxidative stress. Most of the antioxidants have been found to exhibit anticancer and antimutagenic property".*** The presence of allelochemical like polyphenol, flavinoids, isoflavinoids etc. exhibit antioxidant, anti-cancer properties has encouraged us to consider the plant ***Madhuca longifolia*** for exploring anticancer (anti-melanoma) bioefficacy.

PLANT SELECTED FOR STUDY: *MADHUCA LONGIFOLIA*

Habit and Habitat:

Madhuca longifolia is an Indian tropical tree found in the most part of the central and north Indian plains and forests. It is usually known as **mahua**, **mahwa** or **Iluppai**. It is a fast-growing tree that grows just about 20 meters in height, possesses evergreen or semi-evergreen foliage, and belongs to the family **Sapotaceae**. It is adapted to arid environments Maharashtra, Madhya Pradesh, Kerala, Gujarat and Orissa. In India, its two species **(*Madhuca Indica*** *and* ***Madhuca longifolia)*** are found in States of West Bengal, Chhattisgarh, Jharkhand Uttrakhand and Uttar Pradesh *(Yadav et al. 2012, Akshatha et al. 2013, Annalakshmi et al. 2013, Verma et al. 2014, Indus S et al. 2014).*

Classification:

Kingdom: Plante

Family: Sapotaceae

Subfamily: Caesalpinieae

Tribes: Caesalpinieae

Order: Ericaleac

Genus: *Madhuca*

Species: *longifolia*

FIG.5 A *Madhuca longifolia* Plant

Phytochemical screening:

An enthusiastic survey *(Awasthi et al. 1967, kitagawa et al. 1978, Siddique et al. 2007, Khare et al. 2007, Patel et al. 2010, Patel et al. 2012, Triveni et al. 2012, Mishra et al. 2013, Annalakshmi et al. 2013, Verma et al. 2014, Bhaumik et al. 2014, Kamal 2014)* of the literature indicates the presence of a wide range of phytochemicals in various parts of plant as follows. **Plant acids**; Arachidic, linolelic,oleic, myrisic, palmitic and srearic acids, **Plant salts:** Ethylcinnamate, 3β-bmonocaprylic ester of eythrodiol and 3β-capryloxy oleanolic acid and α- and β- amyrin acetates, **Amino acids:** α- alanine, aspartic acid, cystine, glycine, isoleucine and leucine, lysine, methionine, proline, serine, threonine,**Terpenoids**: sesquiterpene, α-terpeneol , β- sitosrerol, **Flavonoids:** myricetin and its 3-O-arabinoside and 3 -O-L-rhamnoside quercetin and its 3-galactoside, quercetin and dihydroquercetin, **Steriods:** stigmasterol, β- si sitosterol, tosterol- β-D-glucoside, **Saponin:** saponin A and B, **Pigments** β-carotene, 3β-caproxyolcan-12-en-28-ol-β-carotene, and xanthophylls, **Vitamins:** Vitamins A and C.

However, a thorough screening of the plant for the presence of other chemicals is essentially required.

Pharmacological properties:

Various parts of the plant have been found to exhibit various pharmacological bioefficacies which are tabulated as follows:

TABLE. 3 PHARMACOLOGICAL PROPERTIES OF THE PLANT *M. longofolia*

Plant Parts	Solvent	Bioefficacy	References
Bark	Ethanol	Antihyperglycemic, Antioxidant Rheumatism activity	*Prashnath et al. 2010*
	Methanol and water	Antidiabetic activity	*Kumar et al. 2011*
	Aq. ethanol acetone	Antibacterial activity	*Tambekar et al. 2010*
	70% Ethanolic extract	Antioxidant activity	*Roy et al. 2010*
	Methanolic extract	Antioxidant activity	*Chaudhary et al. 2012*
	Aq. Methanol extract	Antioxidant activity	*Patil et al. 2009*
	Petroleum ether	Antioxidant activity	*Kausik et al. 2010*
	Ethanol	Antioxidant , Wound healing	*Akshatha et al. 2013*
Leaves	Ethanol	Wound healing	*Marina et al. 2012*
	Aq. Acetone extract	Antioxidant activity	*Kumar et al. 2011*
	Ethanol (90%)	Antioxidant activity	*Palani et al. 2010*
	Methanolic extract	Antioxidant activity	*Inganakal et al. 2013*
	Acetone and ethanol	Cytotoxic activity	*Yadav et al. 2012*
	Ethanol	Nephro and hepato protective activity	*Palani et al. 2010*
	Hydro-ethanolic	Antihyperglycemic activity	*Ghosh et al. 2009*
Flower	Aq. alcoholic extract	Analgesic activity	*Chandra et al. 2001*
	Methanol extract	Hepatoprotective activity	*Palani et al. 2010*
	Juice	Skin spots	*Parasnath et al. 2010*
	Roasted	Cure cough	*Palani et al. 2010*
Seeds	Oil	Pain remover	*Parasnath et al. 2010*
	Aq. alcoholic extract	Analgesic effects	*Chakma et al. 2011*
	Methanol (40-80%)	Oral Pathogens	*Jyothi et al. 2012*

MATERIAL AND METHODS:

Plant species: *Madhuca longifolia*
In vitro: Cell line [B16F10 (Melanoma)] ***In vivo*:** Animal model C57BL/6

Collection and identification of the plant

The plant material of *Madhuca longifolia* (Flowers, leaves and bark) would be collected from the village Rajabarari, Madhya Pradesh. It was identified as *Madhuca longifolia* through Taxonomy, Department of Botany, Dayalbagh Educational Institute, Dayal Bagh Agra.

Extraction of various plant parts

Plant material would be separated (Flowers, leaves and bark) and dried under shade and powered. Each part (500gm) would be separately subjected for the Soxhlet extraction with aqueous-ethanol. In order to remove the chlorophyll, leaves would be extracted in petroleum ether before its extraction in aqueous-ethanolic solvent. Each extract would be concentrated under reduce pressure using Rota vapor vacuum distillation assembly and dried by purging nitrogen (N_2) and weighed.

Phytochemical characterization

The extract of different parts (Flowers, leaves and bark) of the plant *Madhuca longifolia* would be subjected for LC-MS and GC-MS analysis.

Preparation of gold nanoparticles using different plant parts extract

Aqueous sodium tetra chloro aurate ($NaAuCl_4.2H_2O$) solution (1mM) would be added to each extract in the optimized ratio. Sonicated within a particular time span in ultrasonic bath color change would be observed. Gold nanoparticles embedded with plant extract would be characterized by using UV-Visible spectral analysis, Scanning Electron Microscopy, Transmission Electron Microscopy, Atomic Force Microscopy, Zeta potential and X-Rays diffraction.

UV-Visible Spectral analysis

UV-Vis spectroscopy is one of the most important techniques to characterize gold nanoparticles. UV-Vis spectroscopy would be used to observe the SPR band of gold nanoparticles at scanning range between 200-800 nm.

Scanning Electron Microscopy (SEM)

The morphological characteristics of gold nanoparticles can be evaluated using Scanning Electron Microscope.

Transmission Electron Microscopy (TEM)

Transmission Electron Microscopy (TEM) is technique in which a beam of electrons is transmitted through an ultra-thin specimen, interacting with the specimen as it passes through. An image is formed from the interaction of the electrons transmitted through the sample; the image is magnified and focused onto an imaging device. A fluorescent display, on a photographic film is detected by a sensor. TEM is able of imaging at a considerably higher resolution than light microscopes.

Atomic Force Microscopy (AFM)

Atomic Force microscopy (AFM) would be used for gold nanoparticles morphology observation. All scans would be performed in air with commercial Si nanoprobe tips.

Zeta Potential

Zeta potential is a scientific term for electro kinetic potential in colloidal dispersions. It is the potential difference between the dispersion medium and the stationary phase of fluid close to the dispersed particle. It is extensively used for quantification of the magnitude of the charge.

There for, colloids with high zeta potential are electrically stabilized while colloids with low zeta potentials tend to coagulate or flocculate.

X-Ray Diffraction

X-Ray diffraction is a method used for determining the atomic and molecular structure of a crystal in which the crystalline atoms cause a beam of X-rays to diffract into many specific directions. By measuring the angles and intensities of these diffracted beams, a crystallographer can produce a three- dimensional picture of the density of electrons within the crystal.

BIOASSAYS:

In-vitro studies

Appropriate melanoma cell lines (B16F10) would be obtained from National Cell Centre of Science, Pune, and grown in MEM / RPMI 1640 medium supplemented with 1% fetal calf serum (FCS), antibiotic at 37^0C, 5% CO_2 in an incubator for 24 hours.

MTT Assay (Pandey et al. 2006)

The cytotoxic effect of targeted extract against human cancer cell lines/ Animal Cell lines would be determined by a colorimetric assay, by the ability of living cells to reduce the yellow dye MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetraxolium] bromide to a blue formazan product. Cells would be seeded in 96-well microplates and incubated for 24h, at 37^0C, 5% CO_2 humidified. After 24h, the extract dissolved in media would be added in each well and incubated for 3 days (72 hr). Doxorubicin would be used as positive control. First column used as negative control containing no drug and no extract.

(a) Evaluation of Cells Survival

Cell survival would be evaluated by MTT dye which is reduced by the living cells. Absorbance would be determined by ELISA plate reader at 540 nm.

(b) Determination of IC_{50}

The inhibition rate (%) = [1-(Absorbance 540 of sample/Absorbance 540 of control)].

Trypan Blue Exclusion Assay (Frieauff et al. 2001)

Cells would be seeded in tissue culture petri plates and adhere for 24 hr in CO_2 incubator at 37^0C. The medium would be replaced with incomplete MEM medium for 24 hr in CO_2 incubator at 37^0C. Trypan blue dye (0.1 ml, 0.4% in water) would be mixed with cell suspension, 15 min prior to completion of incubation period. At the end of incubation period, the petri plates would be carefully taken out and sodium dodecyal sulfate (1.0%) would be added. Viability would be expressed as a percentage of control number of cells excluding Trypan blue dye.

Fine Needle Aspiration Cytology (Roskell et al. 2004)

Fine needle aspiration cytology (FNAC) involves using a narrow gauge (25-22G) needle to collect a sample of a lesion for microscopic examination. It allows a minimally invasive, rapid diagnosis

of tissue. Aspiration cytology offers a relatively cheap, quick, and accurate tool for the diagnosis and follows up of cancer.

In vivo studies

C57BL/6 mice of 6 weeks of age would be kept in groups five per cage and fed with control diet with water. The animals would be acclimatized for 1 week before use and maintained throughout at standard condition as follows: 24 hr, 22 ± 2^0C temperature, 50% humidity and 12 hr light / dark cycle. Mice would be divided into two main groups normal and cancerous. Cancerous groups would be subdivided into various subgroups: control untreated cancerous animals, animals treated with various plant extracts and animals treated with reference drug (Doxorubicin).

Induction of melanoma cells in mice

At the 4 to 6 weeks of age, 1 x 10^5 viable cells in DMEM medium supplemented with 10% FCS would be induce in the mice.

Administration of test samples

Animals would be treated with the test samples at the first day, after induction of cell line. Another group of animals would be treated with test samples when the tumor becomes 0.5x 0.5cm (*ex-vivo*) or sub-centimetre *in vivo.*

Measurement of tumor volume *(Gupta et al. 2007)*

The volume of the tumor would be recorded by using calipers method and calculated by using the formulae*:* Tumor volume= 4/3 x π x [(½x smaller diameter)^2 x (½ x larger diameter)].

Histological investigation

Sample of tumor would be fixed in 10% Formalin and processed for histology. Tissues embedded in paraffin wax would be sectioned and examined for the architect of the tissues for histological changes if any and blood vessels count.

Chromosomal and micronucleus assay *(Savage et al. 1993* and *Schimid et al. 1975)*

Cytogenetic damage in the bone marrow cells would be studied by chromosomal aberration assay in terms of chromatid break, chromosomal break and asymmetrical exchanges using standard method. Damage induced by the test substance to the chromosomes would be studied by micronucleus method**.**

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